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ORIGINAL ARTICLE

Determining the association between adipokine expression in multiple tissues and phenotypic features of non-alcoholic fatty liver disease in obesity

MGM Wolfs^{1,11}, N Gruben^{1,11}, SS Rensen², FJ Verdam², JW Greve², A Driessen^{3,4}, C Wijmenga⁵, WA Buurman⁶, L Franke^{5,7}, L Scheja⁸, DPY Koonen¹, R Shiri-Sverdlov⁹, TW van Haeften^{1,10}, MH Hofker^{1,11} and J Fu^{5,11}

OBJECTIVES: Non-alcoholic fatty liver disease (NAFLD) is an obesity-associated disease, and in obesity adipokines are believed to be involved in the development of NAFLD. However, it is still not clear whether adipokines in the liver and/or adipose tissues can be related to the development of specific characteristics of NAFLD, such as steatosis and inflammation. We aimed to address this question by simultaneously examining the adipokine expression in three tissue types in obese individuals.

METHODS: We enrolled 93 severely obese individuals with NAFLD, varying from simple steatosis to severe non-alcoholic steatohepatitis. Their expression of 48 adipokines in the liver, visceral and subcutaneous adipose tissue (SAT) was correlated to their phenotypic features of NAFLD. We further determined whether the correlations were tissue specific and/or independent of covariates, including age, sex, obesity, insulin resistance and type 2 diabetes (T2D).

RESULTS: The expression of adipokines showed a liver- and adipose tissue-specific pattern. We identified that the expression of *leptin*, angiopoietin 2 (*ANGPT2*) and *chemerin* in visceral adipose tissue (VAT) was associated with different NAFLD features, including steatosis, ballooning, portal and lobular inflammation. In addition, the expression of tumor necrosis factor (*TNF*), plasminogen activator inhibitor type 1 (*PAI-1*), insulin-like growth factor 1 (somatomedin C) (*IGF1*) and chemokine (C-X-C motif) ligand 10 (*CXCL10*) in the liver tissue and the expression of interleukin 1 receptor antagonist (*IL1RN*) in both the liver and SAT were associated with NAFLD features. The correlations between *ANGPT2* and *CXCL10*, and NAFLD features were dependent on insulin resistance and T2D, but for the other genes the correlation with at least one NAFLD feature remained significant after correcting for the covariates.

CONCLUSIONS: Our results suggest that in obese individuals, VAT-derived *leptin* and *chemerin*, and hepatic expression of *TNF*, *IGF1*, *IL1RN* and *PAI-1* are involved in the development of NAFLD features. Further, functional studies are warranted to establish a causal relationship.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an obesity-associated disease ranging from relatively benign hepatic steatosis to non-alcoholic steatohepatitis (NASH), severe cirrhosis and fibrosis. NASH is characterized by steatosis, hepatic inflammation and cytological ballooning.¹ With the increasing prevalence and severity of obesity and type 2 diabetes (T2D), more patients with NASH will progress to potentially fatal, end-stage liver disease. Specifically, it was shown that almost half of the patients with NASH have advanced fibrosis,² and that 10–25% of the NASH patients will progress to show severe liver pathology such as liver cirrhosis and/or hepatocellular

carcinoma,^{3–6} which are associated with high morbidity and mortality.^{3,5,7}

As NAFLD is strongly associated with obesity and the metabolic syndrome,⁸ one hypothesis is that the altered release of adipokines (signaling molecules from the adipose tissue) that occurs in the metabolic syndrome may be involved in the development of NAFLD.⁹ These adipokines can enter the bloodstream and reach the liver.¹⁰ Adipokines derived from visceral adipose tissue (VAT) may be specifically important, as visceral adiposity is related to obesity-associated co-morbidities such as NAFLD.¹¹ Previous studies have identified an association between elevated plasma levels of the adipokine leptin and NASH in humans.¹² However, as many adipokines are also expressed in the

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liver and other organs,^{13,14} a link between peripheral plasma levels and NASH does not provide evidence that the expression of these adipokines in VAT is associated with NASH.

To date, 48 adipokines have been defined in the literature.^{15–18} However, no systematic analyses have been reported on their role in the development of specific characteristics of NAFLD, such as steatosis and inflammation. In this study, we correlated the expression of 48 adipokines in human hepatic, visceral and subcutaneous adipose tissue (SAT) to specific features of NAFLD in severely obese individuals. We aimed to identify whether adipokines are associated to certain phenotypic features of NAFLD.

SUBJECTS AND METHODS

Study population

Between 2006 and 2009, 93 severely obese individuals with a body mass index (BMI) ranging from 30 to 74 kg m⁻² underwent elective bariatric surgery at the Department of General Surgery, Maastricht University Medical Centre (Maastricht, The Netherlands). Subjects with acute or chronic inflammatory diseases, degenerative diseases and those reporting an alcohol intake exceeding 10 g per day or using anti-inflammatory drugs were excluded. This study was approved by the Medical Ethics Board of Maastricht University Medical Centre and informed written consent was obtained from each individual.

Histological assessment of liver pathology

Wedge liver biopsies were fixed in formalin and embedded in paraffin for histological staining. They were analyzed by an experienced pathologist who was blinded to the clinical and biochemical parameters. Each individual was scored for seven different histological parameters of liver pathology: steatosis, fibrosis, inflammation (lobular inflammation, large lipogranulomas, portal inflammation), liver cell injury (ballooning) and glycogenated nuclei according to the scoring system described by Kleiner *et al.*¹⁹

Tissue sampling, histology preparation and mRNA isolation

Tissue sampling and RNA isolation have been described before.²⁰ In brief, RNA was isolated from VAT, SAT and liver tissue obtained during bariatric surgery with the Qiagen Lipid Tissue Mini Kit (Qiagen, Hilden, Germany, 74804). The Agilent Bioanalyzer (Agilent Technologies, Waldbronn, Germany, 5067–1521) was used to assess RNA quality and concentration.

mRNA profiling and normalization

mRNA pre-hybridization processing and hybridization were performed as described before.^{20,21} Anti-sense RNA synthesis, amplification and purification were performed according to the manufacturer's protocol, using the Ambion Illumina TotalPre Amplification Kit (Applied Biosystems/Ambion, Austin, TX, USA). Complementary RNA was hybridized to microarrays containing 48 755 probes, which targeted 37 776 different genes (Illumina HumanHT12 BeadChips, Illumina, San Diego, CA, USA). These microarrays were scanned on the Illumina BeadArray Reader. After quality control and normalization,²⁰ a total of 82 liver samples, 90 SAT samples and 84 VAT samples were retained for further analysis. The expression data are freely available in the Gene Expression Omnibus (GSE22070).

mRNA analysis of adipokines

To gain insight into the tissue-specific expression of adipokines and their roles in NAFLD, we focused on 69 probes that targeted 48 adipokines, which have been well defined in the literature.^{15–18} If multiple probes were annotated for the same gene, we took the average intensity of these probes per gene. All analyses were done using R (version 2.15.1) (R Foundation for Statistical Computing, Vienna, Austria). The heatmap and hierarchical clustering of adipokine expression in these three tissue samples was plotted using R package Gplots (version 2.11.0). The distance was calculated using the Euclidean distance, and the samples and genes were clustered using the 'complete' method. To identify potentially confounding factors, we calculated Spearman correlation coefficients between phenotypic features of NAFLD and sex, age, BMI, waist-to-hip ratio (WHR), the diagnosis of T2D and the homeostasis model assessment

of insulin resistance (HOMA-IR). In addition, the inter-correlation among NAFLD features and the inter-tissue correlation of adipokine expression levels were computed using the same method (see Supplementary Statistical Notes 1).

To determine how adipokines are involved in NAFLD, we first computed Spearman correlations between liver histology scores and the expression levels of adipokines from each tissue type. The significance of correlation was determined as 7.03×10^{-4} , which corresponds to a false discovery rate of 0.05, using $1000 \times$ permutations (see Supplementary Statistical Notes 2). A partial correlation analysis was performed to correct for inter-tissue correlations of adipokine expression levels and to determine the most relevant tissue type (R package ppcor, version 1.0). To regress out the effect of confounding factors (age, sex, BMI, WHR, T2D and HOMA-IR), we performed conditional regression analysis (see Supplementary Statistical Notes 3).

Plasma measurement of chemerin and ANGPT2

For determination of plasma adipokine levels, venous blood samples were collected in EDTA tubes after an 8-h fasting period. Plasma was used to measure chemerin and angiopoietin 2 (ANGPT2) levels using commercially available enzyme-linked immunosorbent assay (R&D Systems, Abingdon, UK). A Spearman correlation analysis was used to test the correlation between plasma levels, gene expression and different NAFLD features.

RESULTS

Severely obese individuals show varying degrees of NAFLD

Plasma parameters and clinical traits of all the subjects are shown in Supplementary Table 1. We scored 7 histological characteristics of NAFLD in 93 severely obese individuals, following Kleiner *et al.*'s method.¹⁹ Of these individuals, 25 showed no signs of NAFLD, while 8 showed simple steatosis without inflammation. The other 60 individuals had steatosis accompanied by varying degrees of inflammation (Table 1).

NAFLD characteristics correlated with other phenotypes and adipokine expression shows inter-tissue correlations

No strong associations between NAFLD features and sex or age were observed (Figure 1a). In line with clinical observations,⁸ the NAFLD features steatosis, fibrosis, ballooning and inflammation were correlated with BMI, WHR, T2D and HOMA-IR (Figure 1a). Many NAFLD features also showed significant inter-correlations. For example, in accordance with its central role in NAFLD, steatosis was correlated with all other NAFLD features scored (Figure 1b).

Hierarchical clustering of samples based on the genome-wide gene expression in the liver, SAT and VAT (Supplementary Figure 1) showed that sample clusters did not correspond to any cofactors or NAFLD features. Thus, general differences in gene expression in

Table 1. Summary of liver pathology of the study population

Features	Individuals scored (number)	Grade (number of individuals)			
		0	1	2	3
Steatosis (0–3)	93	25	26	29	13
Fibrosis (0–3)	91	62	15	11	3
Lobular inflammation (0–3)	88	36	35	12	5
Large lipogranulomas (0–1)	92	70	22	—	—
Portal inflammation (0–1)	93	72	21	—	—
Ballooning (0–2)	92	49	39	4	—
Glycogenated nuclei (0–1)	90	74	16	—	—

The data shown are the total number of individuals scored for each marker and the number of individuals with different grades for the seven features of non-alcoholic fatty liver disease, as described in (ref. 19).

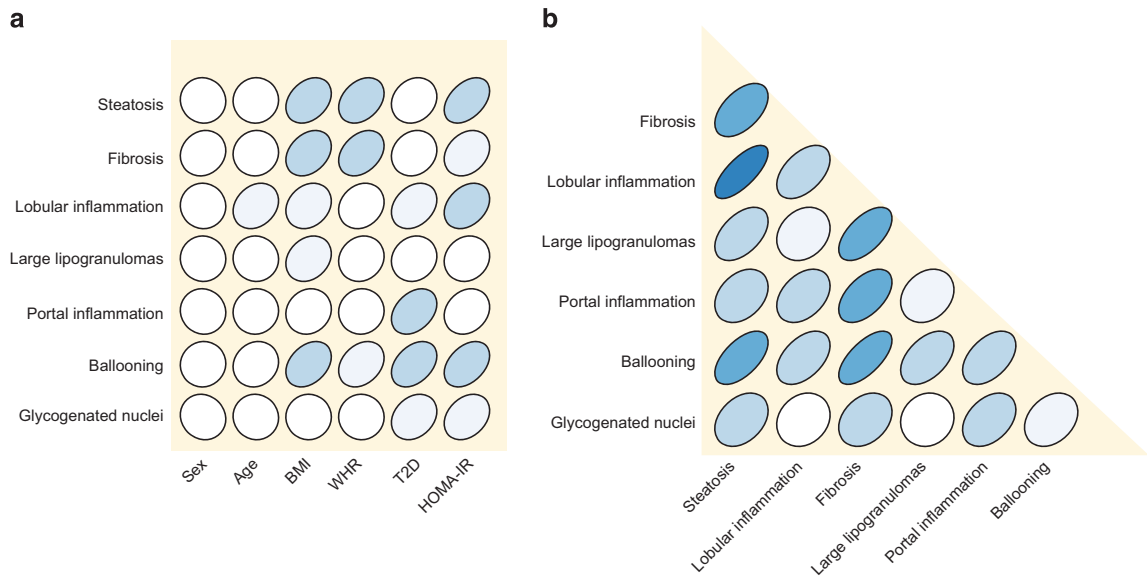


Figure 1. Correlations of features of NAFLD. **(a)** The correlation between the NAFLD features and sex, age, BMI, WHR, T2D and HOMA-IR. **(b)** The inter-correlation among the NAFLD features. The correlations were computed with Spearman correlation and confidence ellipses serve as visual indicators of correlation. All correlations significant at $P \leq 0.05$ are indicated in blue. The darker shade of blue indicates a stronger correlation.

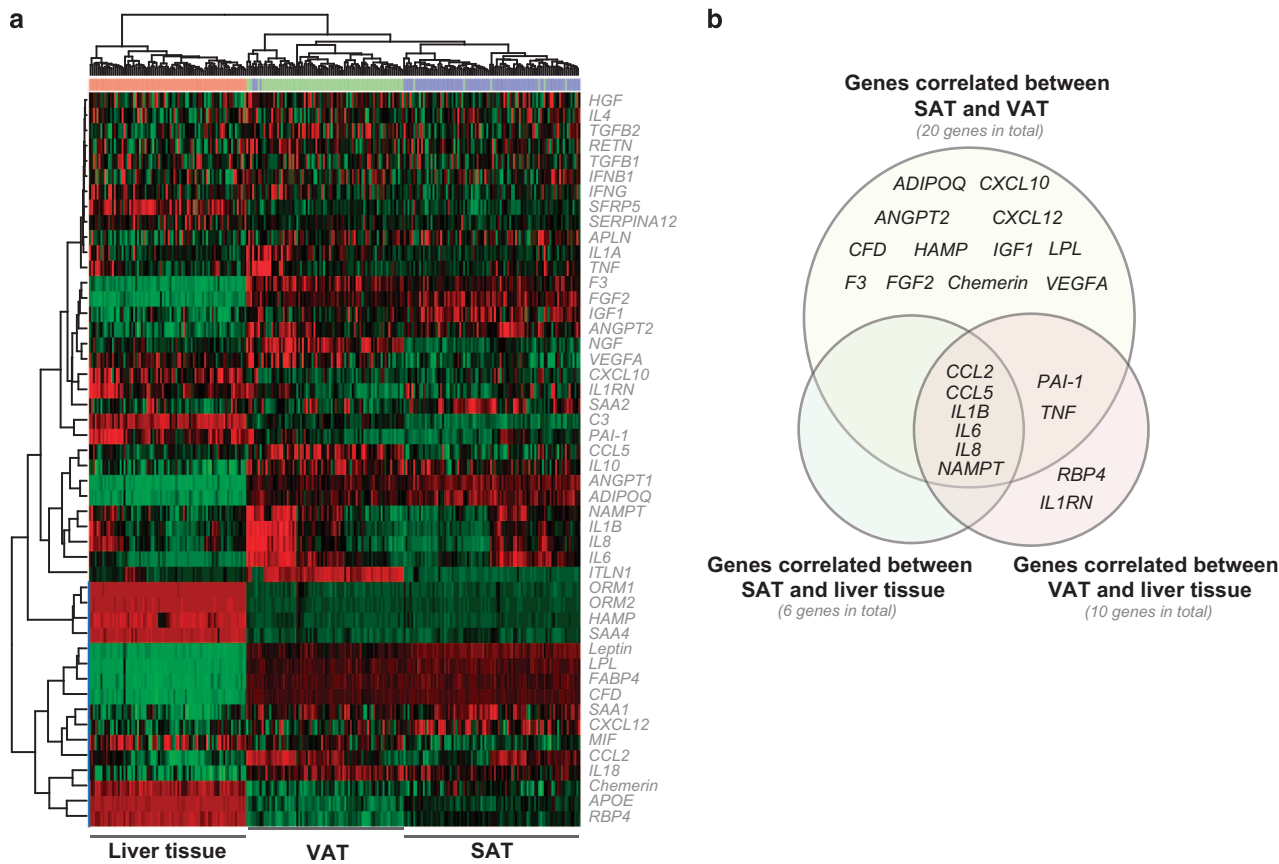


Figure 2. The expression of adipokines in liver tissue, subcutaneous and VAT. **(a)** The heatmap diagram shows the differential gene expression in joints from the three tissue types. The tissue samples (top row: red, liver samples; green, VAT samples; blue, SAT samples) and genes (left side) are clustered hierarchically. Each column represents a tissue sample and each row represents the expression of a single gene (green, low expression; red, high expression). **(b)** Venn diagram showing correlation of gene expression between tissues at false discovery rate = 0.05.

Table 2. The adipokines that were correlated with features of NAFLD

Gene	Feature	Tissue	Not corrected	Tissue corrected	Age, sex corrected	Age, sex, obesity corrected	Age, sex, obesity, T2D and IR corrected
			Correlation P-value	Correlation P-value	Correlation P-value	Correlation P-value	Correlation P-value
<i>ANGPT2</i>	Lobular inflam.	VAT	0.42 $P=1.1 \times 10^{-4}$	0.36 $P=2.1 \times 10^{-3}$	0.45 $P=3.1 \times 10^{-5}$	0.40 $P=0.0027$	0.24 $P=0.11$
<i>Leptin</i>	Lobular inflam.	VAT	0.39 $P=4.0 \times 10^{-4}$	0.34 $P=3.3 \times 10^{-3}$	0.406 $P=2.1 \times 10^{-4}$	0.33 $P=0.016$	0.28 $P=0.05$
	Steatosis	VAT	0.41 $P=9.4 \times 10^{-5}$	0.40 $P=2.5 \times 10^{-4}$	0.454 $P=1.46 \times 10^{-5}$	0.038 $P=0.781$	-0.031 $P=0.83$
<i>Chemerin</i>	Lobular inflam.	VAT	-0.51 $P=1.5 \times 10^{-6}$	-0.41 $P=3.0 \times 10^{-4}$	-0.552 $P=1.33 \times 10^{-7}$	-0.54 $P=2.9 \times 10^{-5}$	-0.49 $P=5.1 \times 10^{-4}$
	Lobular inflam.	SAT	-0.37 $P=5.6 \times 10^{-4}$	-0.17 $P=0.17$	-0.347 $P=0.00114$	-0.32 $P=0.016$	-0.22 $P=0.13$
	Portal inflam.	VAT	-0.37 $P=5.9 \times 10^{-4}$	-0.31 $P=7.2 \times 10^{-3}$	-0.375 $P=4.5 \times 10^{-3}$	-0.34 $P=0.0092$	-0.28 $P=0.05$
	Steatosis	VAT	-0.39 $P=2.9 \times 10^{-4}$	-0.24 $P=0.037$	-0.39 $P=2.1 \times 10^{-3}$	-0.32 $P=0.015$	-0.31 $P=0.03$
<i>IL1RN</i>	Lobular inflam.	Liver	0.46 $P=2.2 \times 10^{-5}$	0.38 $P=9.1 \times 10^{-4}$	0.44 $P=4.9 \times 10^{-5}$	0.45 $P=4.5 \times 10^{-4}$	0.40 $P=0.0038$
	Ballooning	SAT	0.39 $P=1.4 \times 10^{-4}$	0.41 $P=2.4 \times 10^{-3}$	0.38 $P=2.6 \times 10^{-4}$	0.42 $P=9.2 \times 10^{-4}$	0.32 $P=0.019$
<i>CXCL10</i>	Steatosis	Liver	0.38 $P=4.9 \times 10^{-4}$	0.35 $P=1.7 \times 10^{-3}$	0.38 $P=5.2 \times 10^{-4}$	0.27 $P=0.036$	0.25 $P=0.069$
	Fibrosis	Liver	0.40 $P=2.9 \times 10^{-4}$	0.33 $P=3.5 \times 10^{-3}$	0.40 $P=2.0 \times 10^{-4}$	0.19 $P=0.16$	0.21 $P=0.145$
<i>IGF1</i>	Steatosis	Liver	-0.41 $P=1.5 \times 10^{-4}$	-0.37 $P=9.8 \times 10^{-4}$	-0.43 $P=4.8 \times 10^{-5}$	-0.32 $P=0.012$	-0.29 $P=0.037$
<i>PAI-1</i>	Lobular inflam.	Liver	0.55 $P=2.5 \times 10^{-7}$	0.42 $P=2.0 \times 10^{-4}$	0.52 $P=1.1 \times 10^{-6}$	0.49 $P=9.4 \times 10^{-5}$	0.43 $P=0.0018$
	Portal inflam.	Liver	0.37 $P=6.6 \times 10^{-4}$	0.25 $P=0.028$	0.38 $P=4.8 \times 10^{-4}$	0.46 $P=1.7 \times 10^{-4}$	0.39 $P=0.0034$
<i>TNF</i>	Lobular inflam.	Liver	0.38 $P=7.0 \times 10^{-4}$	0.29 $P=0.014$	0.34 $P=0.0027$	0.37 $P=0.044$	0.38 $P=0.0063$

Abbreviations: BMI, body mass index; FDR, false discovery rate; HOMA-IR, homeostasis model assessment of insulin resistance; Inflam., inflammation; NAFLD, non-alcoholic fatty liver disease; SAT, subcutaneous adipose tissue; T2D, type 2 diabetes; VAT, visceral adipose tissue; WHR, waist-to-hip ratio. The table shows all the adipokines that were correlated with liver histology (FDR < 0.05) and the correlation adjusted for tissue type and covariates, including age, sex, BMI, WHR, HOMA-IR and T2D. Bold values indicate the correlations that were not significant at $P \leq 0.05$ after correction.

obese individuals cannot explain a possible association between adipokine expression and NAFLD features (Supplementary Figure 1).

Most of the adipokines showed tissue-dependent expression, which allowed us to classify tissue samples based on their expression profile (Figure 2a). Of the 48 adipokines tested, 26 did not show inter-tissue correlation, but 22 adipokines showed expression levels that were correlated between two or three tissues (Figure 2b). Twenty adipokines showed an inter-tissue correlation between VAT and SAT, and six adipokines showed an inter-tissue correlation between SAT and hepatic tissue. The expression of 10 adipokines was correlated between VAT and hepatic tissue (Figure 2b).

The expression of eight adipokines was associated with NAFLD features

We observed that the expression levels of eight adipokines (angiopoietin 2 (*ANGPT2*), *leptin*, *chemerin*, tumor necrosis factor (*TNF*), interleukin (IL)-1 receptor antagonist (*IL1RN*), chemokine (C-X-C motif) ligand 10 (*CXCL10*), insulin-like growth factor 1 (somatomedin C) (*IGF1*) and plasminogen activator inhibitor type 1 (*PAI-1*)) in one or more tissue types correlated with steatosis, ballooning, lobular inflammation, portal inflammation and/or fibrosis at $P < 7.0 \times 10^{-4}$ (false discovery rate < 0.05) (Table 2 and Figure 3). Large lipogranulomas and glycogenated nuclei did not correlate with adipokine expression levels. As the expression

levels of many adipokines showed inter-tissue correlations (Figure 2b), we performed a partial correlation analysis to correct for the impact of the other tissues. This allowed us to identify the most relevant tissue type associated to NAFLD. The expression of *chemerin* in both SAT and VAT was negatively correlated with lobular inflammation. Taking into account the co-expression of *chemerin* in both adipose tissues (Figure 2b), the partial correlation analysis showed that only *chemerin* expression in VAT was associated with lobular inflammation (Table 2). In this way, we identified three adipokines (*ANGPT2*, *leptin* and *chemerin*) whose expression in VAT was associated to lobular inflammation, ballooning and/or steatosis. In addition, the expression of *TNF*, *IL1RN*, *CXCL10*, *IGF1* and *PAI-1* in the liver, and the expression of *IL1RN* in SAT, were correlated to NAFLD features (Table 2).

Of the individuals in our cohort, 67 out of 93 (72%) were women with age ranging between 17 and 65 years, including 13 women aged over 50 years, some of whom might be post menopausal. The menopausal status may have an effect on cytokine expression and NAFLD development, but we had no information on their menopausal status. We therefore first investigated the correlation between adipokines and NAFLD features by correcting for age and gender. After correcting for this, all the detected correlations remained significant (Table 2). Next, we confined the analysis to 54 women under 50 years old and observed the same effects (Supplementary Table 2). These results suggest that the

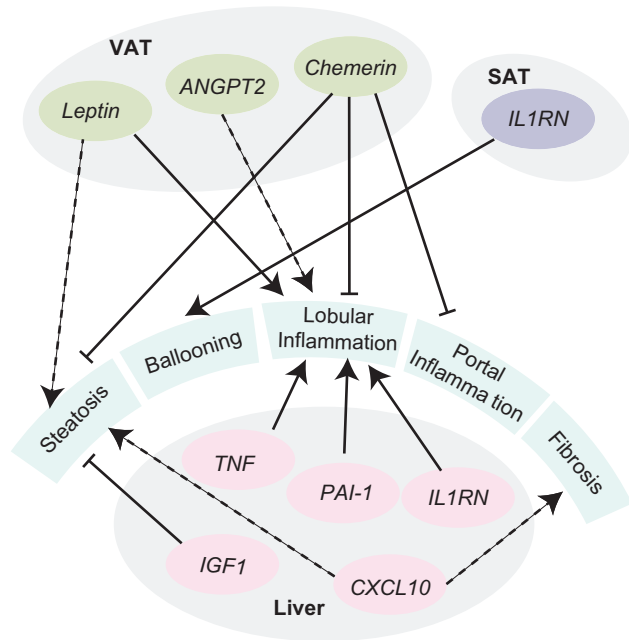


Figure 3. The tissue-specific correlation between adipokine expression and liver histology. Each gene node indicates the expression of a gene in a certain tissue type and each line indicates a significant correlation with liver histology after correction for expression in other tissue types. The arrowhead indicates a positive correlation and the bar head a negative correlation ($P < 0.05$). The dashed line indicates a correlation that is dependent on covariates, including age, sex, BMI, WHR, T2D and/or HOMA-IR.

correlations detected between adipokines and NAFLD development are independent of age, sex and menopausal status.

NAFLD is an obesity-related disease and shows high comorbidity with other obesity-related complications, such as insulin resistance and T2D. Our data also showed that BMI, WHR, T2D and HOMA-IR were correlated with several NAFLD features (Figure 1a). We therefore examined whether the correlation between adipokine expression and NAFLD features was dependent on these confounders. After step-wise correction for the effect of age, sex, obesity and diabetes parameters, the correlations between *leptin* expression in VAT and steatosis were no longer significant. In addition, the correlation for *CXCL10* expression in liver and *ANGPT2* expression in VAT disappeared (Table 2), which indicates that these correlations were largely dependent on the obesity and other obesity-related disorders. Nevertheless, the other correlations were still significant at $P < 0.05$ after this correction (Figure 3 and Table 2).

Peripheral plasma chemerin and ANGPT2 levels do not correlate with NAFLD features

Adipokines derived from VAT may be important in the etiology of NAFLD, as visceral adiposity is mainly related to obesity-associated NAFLD.¹¹ As adipokines expressed in adipose tissue are expected to have endocrine effects on the liver through secretion in the bloodstream, we measured plasma chemerin and ANGPT2 levels (Supplementary Table 1). However, we found no evidence that plasma chemerin and ANGPT2 levels were associated to NAFLD features (Table 3). Moreover, plasma chemerin levels did not correlate with *chemerin* expression in any of the three tissues. Only hepatic expression of *ANGPT2* correlated with plasma ANGPT2 levels (Table 3).

Table 3. Correlation between plasma adipokine levels and tissue expression levels or features of non-alcoholic fatty liver disease

	ANGPT2		Chemerin	
	Correlation	P-value	Correlation	P-value
<i>Expression levels</i>				
Liver	0.313*	0.035	0.171	0.255
SAT	0.225	0.128	0.098	0.511
VAT	0.198	0.198	0.066	0.671
<i>Features</i>				
Steatosis	0.098	0.501	−0.072	0.622
Fibrosis	0.201	0.170	−0.275	0.058
Lobular inflammation	0.028	0.852	−0.118	0.431
Large lipogranulomas	−0.005	0.973	−0.174	0.236
Portal inflammation	0.148	0.309	0.152	0.296
Ballooning	0.239	0.102	−0.184	0.209
Glycogenated nuclei	−0.052	0.730	−0.099	0.509

Abbreviations: ANGPT2, angiopoietin 2; NAFLD, non-alcoholic fatty liver disease; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue. Correlation coefficients of plasma adipokine levels and tissue expression levels or features of NAFLD are shown, with their respective P -value. The significance threshold for these correlation coefficients was 0.28 ($*P < 0.05$).

DISCUSSION

We have simultaneously assessed the expression of 48 adipokines in the liver, VAT and SAT, to identify which adipokines in which tissues were associated with specific features of NAFLD. We observed that the hepatic expression of five genes, including *TNF*, *IGF1*, *IL1RN*, *PAI-1* and *CXCL10*, was correlated with NAFLD features. These correlations were independent of obesity and T2D, except for *CXCL10*. The central role of these adipokines in the progression of NAFLD is supported by many publications. *TNF* activates c-Jun NH₂-terminal kinase and inhibitor of κ B-kinase- β , thus resulting in increased production of additional inflammatory cytokines.²² In addition, enhanced *TNF* signaling was reported to aggravate hepatic inflammation and fibrosis in mice.²³ These findings are in line with the positive association we found between *TNF* expression in the liver and lobular inflammation. *IL1RN* is an antagonist of the IL-1 receptor and reduces the inflammation-related activities of IL-1A and 1B.²⁴ *IL1RN* serum levels as well as liver mRNA expression have been correlated to features of the metabolic syndrome, including NASH.^{25,26} The latter findings are confirmed by our study. *CXCL10* stimulates monocyte and T-cell migration.²⁷ Hepatic *CXCL10* mRNA expression is associated with the presence of necro-inflammatory foci in the liver in mice on a methionine- and choline-deficient diet.²⁸ Our data now add that hepatic *CXCL10* expression is also associated with the development of fibrosis and steatosis in human NASH. However, we showed that this correlation was dependent on morbid obesity and related disorders. Thus, its role in the development of NAFLD needs further investigation in a larger cohort of NAFLD patients, with and without other obesity disorders. *IGF1* has a similar function to insulin. *IGF1* plasma levels are decreased in individuals with hepatic steatosis²⁹ and hepatic mRNA expression of *IGF1* is negatively correlated to fibrosis.³⁰ In line with this, we observed a negative correlation between *IGF1* expression in the liver and steatosis. *PAI-1* is known to be an inhibitor of fibrinolysis.³¹ Hepatic gene expression and plasma *PAI-1* levels are higher in individuals with NAFLD and NASH compared with healthy controls, and increase in parallel with the severity of NAFLD features, including lobular inflammation.³² This was confirmed by our findings of a strong positive correlation between hepatic *PAI-1*

expression and lobular inflammation. Inhibition of fibrinolysis by PAI-1 may cause lobular inflammation due to tissue damage induced by increased fibrin deposition.

Interestingly, our study showed that the expression levels of three genes in VAT (*leptin*, *chemerin* and *ANGPT2*) were associated with NAFLD features. This reinforces the detrimental role of VAT in liver diseases.¹¹ Leptin regulates food intake and energy expenditure, and is elevated in obesity.³³ However, the role of *leptin* in the development of NASH has been controversial. Several studies have shown that plasma *leptin* levels are elevated in patients with NASH³⁴ and are correlated with steatosis,¹² whereas others did not find any association.^{35,36} The positive association we found between *leptin* expression in VAT and lobular inflammation after adjusting for age, gender, BMI and WHR, suggests that *leptin* contributes to the development of hepatic inflammation in NAFLD. This is in line with the previous findings that leptin is also important in regulating immune function.³⁷ We observed that the *ANGPT2* expression in VAT is related to lobular inflammation in NASH, although we also showed that this correlation was dependent on the presence of insulin resistance and/or T2D. This is in line with previous research that showed elevated ANPGT2 plasma levels in obese and T2D patients.^{38–40}

The most striking observation of our study was that *chemerin* expression in VAT was negatively correlated to steatosis, lobular inflammation and portal inflammation independent of obesity, T2D and HOMA-IR. The literature reports conflicting evidence on the role of chemerin in inflammation and NAFLD. Mice lacking the chemerin receptor, chemokine-like receptor 1, were resistant to central nervous system inflammation,⁴¹ but more susceptible to inflammation in models of inflammatory pulmonary disease.^{42,43} In addition, these mice were reported to have reduced hepatic steatosis and hepatic inflammation in one study,⁴⁴ whereas this was not affected in another study.⁴⁵ Our study now suggests that increased *chemerin* expression in VAT may reduce hepatic inflammation. We further investigated whether this association can be explained by endocrine effects, as adipokines are secreted into the bloodstream from adipose tissue. However, in contrast to earlier studies,^{46,47} chemerin plasma levels did not correlate with NAFLD features in our study nor with chemerin expression levels in the three tissues under study (Table 3). Further analyses are needed to investigate the underlying mechanisms behind the negative correlation between chemerin expression in VAT and hepatic inflammation.

In conclusion, our results suggest that *leptin*, *chemerin*, *ANGPT2*, *TNF*, *CXCL10*, *IGF1*, *IL1RN* and *PAI-1* could have a role in the development of specific features of the NAFLD phenotype. Most of the observed effects were independent of age, sex, obesity, insulin resistance and T2D. As it is not known why some patients with fatty liver progress towards hepatic inflammation while others do not, it is tempting to suggest that adipokines have a role in this process. Further functional studies with gene knockout animal models and tissue culture experiments may provide more information on the association we have discovered between adipokines and specific features of the NAFLD phenotype.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

- Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. *Science* 2011; **332**: 1519–1523.
- Dixon JB, Bhathal PS, O'Brien PE. Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. *Gastroenterology* 2001; **121**: 91–100.
- Adams LA, Lymp JF St, Sauver J, Sanderson SO, Lindor KD, Feldstein A et al. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 2005; **129**: 113–121.
- Adams LA, Sanderson S, Lindor KD, Angulo P. The histological course of non-alcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 2005; **42**: 132–138.
- Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413–1419.
- Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010; **51**: 1820–1832.
- Sanyal AJ American Gastroenterological Association. AGA technical review on nonalcoholic fatty liver disease. *Gastroenterology* 2002; **123**: 1705–1725.
- Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 2003; **37**: 917–923.
- Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010; **52**: 1836–1846.
- Wiest R, Moleda L, Farkas S, Scherer M, Kopp A, Wonckhaus U et al. Splanchnic concentrations and postprandial release of visceral adipokines. *Metabolism* 2010; **59**: 664–670.
- Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *Eur Heart J* 2008; **29**: 2959–2971.
- Chitturi S, Farrell G, Frost L, Kriketos A, Lin R, Fung C et al. Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: a manifestation of lipotoxicity? *Hepatology* 2002; **36**: 403–409.
- Chamberland JP, Berman RL, Aronis KN, Mantzoros CS. Chemerin is expressed mainly in pancreas and liver, is regulated by energy deprivation, and lacks day/night variation in humans. *Eur J Endocrinol* 2013; **169**: 453–462.
- Dominguez M, Miquel R, Colmenero J, Moreno M, Garcia-Pagan JC, Bosch J et al. Hepatic expression of CXC chemokines predicts portal hypertension and survival in patients with alcoholic hepatitis. *Gastroenterology* 2009; **136**: 1639–1650.
- Deng Y, Scherer PE. Adipokines as novel biomarkers and regulators of the metabolic syndrome. *Ann N Y Acad Sci* 2010; **1212**: E1–E19.
- Juge-Aubry CE, Henrichot E, Meier CA. Adipose tissue: a regulator of inflammation. *Best Pract Res Clin Endocrinol Metab* 2005; **19**: 547–566.
- Wozniak SE, Gee LL, Wachtel MS, Frezza EE. Adipose tissue: the new endocrine organ? A review article. *Dig Dis Sci* 2009; **54**: 1847–1856.
- Lonardo A, Carani C, Carulli N, Loria P. 'Endocrine NAFLD' a hormonocentric perspective of nonalcoholic fatty liver disease pathogenesis. *J Hepatol* 2006; **44**: 1196–1207.
- Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313–1321.
- Wolfs MG, Rensen SS, Bruin-Van Dijk EJ, Verdam FJ, Greve JW, Sanjabi B et al. Co-expressed immune and metabolic genes in visceral and subcutaneous adipose tissue from severely obese individuals are associated with plasma HDL and glucose levels: a microarray study. *BMC Med Genomics* 2010; **3**: 34.
- Fu J, Wolfs MG, Deelen P, Westra HJ, Fehrmann RS, Te Meerman GJ et al. Unraveling the regulatory mechanisms underlying tissue-dependent genetic variation of gene expression. *PLoS Genet* 2012; **8**: e1002431.
- Carter-Kent C, Zein NN, Feldstein AE. Cytokines in the pathogenesis of fatty liver and disease progression to steatohepatitis: implications for treatment. *Am J Gastroenterol* 2008; **103**: 1036–1042.
- Aparicio-Vergara M, Hommelberg PP, Schreurs M, Gruben N, Stienstra R, Shiri-Sverdlor R et al. Tumor necrosis factor receptor 1 gain-of-function mutation aggravates nonalcoholic fatty liver disease but does not cause insulin resistance in a murine model. *Hepatology* 2013; **57**: 566–576.
- Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 2009; **27**: 519–550.
- Pihlajamäki J, Kuulasmaa T, Kaminska D, Simonen M, Karja V, Gronlund S et al. Serum interleukin 1 receptor antagonist as an independent marker of non-alcoholic steatohepatitis in humans. *J Hepatol* 2012; **56**: 663–670.

- 26 Estep JM, Baranova A, Hossain N, Elariny H, Ankrah K, Afendy A *et al*. Expression of cytokine signaling genes in morbidly obese patients with non-alcoholic steatohepatitis and hepatic fibrosis. *Obes Surg* 2009; **19**: 617–624.
- 27 Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo JR, Harada A *et al*. Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J Exp Med* 1993; **177**: 1809–1814.
- 28 Maina V, Sutti S, Locatelli I, Vidali M, Mombello C, Bozzola C *et al*. Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice. *Clin Sci (Lond)* 2012; **122**: 545–553.
- 29 Volzke H, Nauck M, Rettig R, Dorr M, Higham C, Brabant G *et al*. Association between hepatic steatosis and serum IGF1 and IGFBP-3 levels in a population-based sample. *Eur J Endocrinol* 2009; **161**: 705–713.
- 30 Hribal ML, Procopio T, Petta S, Sciacqua A, Grimaudo S, Pipitone RM *et al*. Insulin-like growth factor-I, inflammatory proteins, and fibrosis in subjects with nonalcoholic fatty liver disease. *J Clin Endocrinol Metab* 2013; **98**: E304–E308.
- 31 Cesari M, Pahor M, Incalzi RA. Plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. *Cardiovasc Ther* 2010; **28**: e72–e91.
- 32 Verrijken A, Franque S, Mertens I, Prawitt J, Caron S, Hubens G *et al*. Prothrombotic factors in histologically proven NAFLD and NASH. *Hepatology* 2013. e-pub ahead of print 23 May 2013; doi:10.1002/hep.26510.
- 33 Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998; **395**: 763–770.
- 34 Uygun A, Kadayifci A, Yesilova Z, Erdil A, Yaman H, Saka M *et al*. Serum leptin levels in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 2000; **95**: 3584–3589.
- 35 Kashyap SR, Diab DL, Baker AR, Yerian L, Bajaj H, Gray-McGuire C *et al*. Triglyceride levels and not adipokine concentrations are closely related to severity of nonalcoholic fatty liver disease in an obesity surgery cohort. *Obesity (Silver Spring)* 2009; **17**: 1696–1701.
- 36 Chalasani N, Crabb DW, Cummings OW, Kwo PY, Asghar A, Pandya PK *et al*. Does leptin play a role in the pathogenesis of human nonalcoholic steatohepatitis? *Am J Gastroenterol* 2003; **98**: 2771–2776.
- 37 Carlton ED, Demas GE, French SS. Leptin, a neuroendocrine mediator of immune responses, inflammation, and sickness behaviors. *Horm Behav* 2012; **62**: 272–279.
- 38 Lieb W, Zachariah JP, Xanthakis V, Safa R, Chen MH, Sullivan LM *et al*. Clinical and genetic correlates of circulating angiopoietin-2 and soluble Tie-2 in the community. *Circ Cardiovasc Genet* 2010; **3**: 300–306.
- 39 Lim HS, Blann AD, Chong AY, Freestone B, Lip GY. Plasma vascular endothelial growth factor, angiopoietin-1, and angiopoietin-2 in diabetes: implications for cardiovascular risk and effects of multifactorial intervention. *Diabetes Care* 2004; **27**: 2918–2924.
- 40 Silha JV, Krsek M, Sucharda P, Murphy LJ. Angiogenic factors are elevated in overweight and obese individuals. *Int J Obes (Lond)* 2005; **29**: 1308–1314.
- 41 Graham KL, Zabel BA, Loghavi S, Zuniga LA, Ho PP, Sobel RA *et al*. Chemokine-like receptor-1 expression by central nervous system-infiltrating leukocytes and involvement in a model of autoimmune demyelinating disease. *J Immunol* 2009; **183**: 6717–6723.
- 42 Bondue B, Vosters O, de Nadai P, Glineur S, De Henau O, Luangsang S *et al*. ChemR23 dampens lung inflammation and enhances anti-viral immunity in a mouse model of acute viral pneumonia. *PLoS Pathog* 2011; **7**: e1002358.
- 43 Luangsang S, Wittamer V, Bondue B, De Henau O, Rouger L, Brait M *et al*. Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. *J Immunol* 2009; **183**: 6489–6499.
- 44 Ernst MC, Haidl ID, Zuniga LA, Dranse HJ, Rourke JL, Zabel BA *et al*. Disruption of the chemokine-like receptor-1 (CMKLR1) gene is associated with reduced adiposity and glucose intolerance. *Endocrinology* 2012; **153**: 672–682.
- 45 Gruben N, Aparicio Vergara M, Kloosterhuis NJ, van der Molen H, Stoelwinder S, Youssef S *et al*. Chemokine-like receptor 1 deficiency does not affect the development of insulin resistance and nonalcoholic fatty liver disease in mice. *PLoS One* 2014; **9**: e96345.
- 46 Kukla M, Zwirska-Korcza K, Hartleb M, Waluga M, Chwist A, Kajor M *et al*. Serum chemerin and vaspin in non-alcoholic fatty liver disease. *Scand J Gastroenterol* 2010; **45**: 235–242.
- 47 Sell H, Divoux A, Poitou C, Basdevant A, Bouillot JL, Bedossa P *et al*. Chemerin correlates with markers for fatty liver in morbidly obese patients and strongly decreases after weight loss induced by bariatric surgery. *J Clin Endocrinol Metab* 2010; **95**: 2892–2896.



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